

# Phosphatidylinositol 3-Kinase-dependent Membrane Association of the Bruton's Tyrosine Kinase Pleckstrin Homology Domain Visualized in Single Living Cells\*

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Phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P<sub>3</sub>) has been proposed to act as a second messenger to recruit regulatory proteins to the plasma membrane via their pleckstrin homology (PH) domains. The PH domain of Bruton's tyrosine kinase (Btk), which is mutated in the human disease X-linked agammaglobulinemia, has been shown to interact with PI(3,4,5)P<sub>3</sub> *in vitro*. In this study, a fusion protein containing the PH domain of Btk and the enhanced green fluorescent protein (BtkPH-GFP) was constructed and utilized to study the ability of this PH domain to interact with membrane inositol phospholipids inside living cells. The localization of expressed BtkPH-GFP in quiescent NIH 3T3 cells was indistinguishable from that of GFP alone, both being cytosolic as assessed by confocal microscopy. In NIH 3T3 cells coexpressing BtkPH-GFP and the epidermal growth factor receptor, activation of epidermal growth factor or endogenous platelet-derived growth factor receptors caused a rapid (<3 min) translocation of the cytosolic fluorescence to ruffle-like membrane structures. This response was not observed in cells expressing GFP only and was completely inhibited by treatment with the PI 3-kinase inhibitors wortmannin and LY 292004. Membrane-targeted PI 3-kinase also caused membrane localization of BtkPH-GFP that was slowly reversed by wortmannin. When the R28C mutation of the Btk PH domain, which causes X-linked agammaglobulinemia, was introduced into the fluorescent construct, no translocation was observed after stimulation. In contrast, the E41K mutation, which confers transforming activity to native Btk, caused significant membrane localization of BtkPH-GFP with characteristics indicating its possible binding to PI(4,5)P<sub>2</sub>. This mutant, but not wild-type BtkPH-GFP, interfered with agonist-induced PI(4,5)P<sub>2</sub> hydrolysis in COS-7 cells. These results show in intact cells that the PH domain of Btk binds selectively to 3-phosphorylated lipids after activation of PI 3-kinase enzymes and that losing such binding ability or specificity results in gross abnormalities in the function of the enzyme. Therefore, the interaction with PI(3,4,5)-P<sub>3</sub> is likely to be an important determinant of the physiological regulation of Btk and can be utilized to visualize the dynamics and spatiotemporal organization of changes in this phospholipid in living cells.

Phosphoinositides are important precursor molecules that generate multiple second messengers in stimulated cells. Phospholipase C (PLC)<sup>1</sup>-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub>) yields the water-soluble messenger molecule inositol 1,4,5-trisphosphate, which mobilizes intracellular Ca<sup>2+</sup>, and the hydrophobic moiety diacylglycerol, which activates protein kinase C isozymes (1, 2). In addition, PI(4,5)P<sub>2</sub> can be phosphorylated by PI 3-kinase(s) to form phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P<sub>3</sub>), a lipid product found only in stimulated cells (3, 4). The multiplicity and divergent regulation of PI 3-kinases by receptor and non-receptor tyrosine kinases as well as by GTP-binding proteins (5) and the resistance of PI(3,4,5)P<sub>3</sub> to hydrolysis by any known phospholipase C have led to the proposal that 3-phosphorylated inositides, in particular PI(3,4,5)P<sub>3</sub>, serve important regulatory functions (5).

The pleckstrin homology (PH) domains of several regulatory proteins have been shown to bind PI(3,4,5)P<sub>3</sub> *in vitro* (6–10). One of these proteins is Bruton's tyrosine kinase (Btk), a member of the Tec family of non-receptor tyrosine kinases (11), mutations of which are associated with the human disease X-linked agammaglobulinemia and its murine equivalent, X-linked immunodeficiency (12, 13). Although Btk also contains a protein kinase as well as SH2 and SH3 domains, its PH domain alone has been shown to be sufficient to bind PI(3,4,5)P<sub>3</sub> selectively *in vitro* (6, 8). Many of the Btk mutations that cause the B-cell defect that leads to X-linked agammaglobulinemia in humans (14) are within the Btk PH domain of the protein, and one of these, the R28C substitution, is responsible for X-linked immunodeficiency in mice (13). The latter mutation has also been shown to abolish the binding of Btk to inositol lipids *in vitro* (6, 8). In addition, a transforming mutant of Btk (Btk\*, E41K) has been reported to show increased membrane association, which further indicates that PH domain-mediated binding of Btk to cell membrane(s) is critical for its activation (15).

This study was designed to investigate whether the isolated PH domain of Btk is sufficient to interact with membrane phosphoinositides within intact living cells with similar specificity to that described *in vitro* and whether this interaction can localize the protein to the membrane without additional binding motifs. Expression of the Btk PH domain fused to the enhanced green fluorescent protein (BtkPH-GFP) has demonstrated that PI 3-kinase activation recruits these molecules to the plasma membrane, suggesting that they specifically recognize 3-phosphorylated inositol lipids without binding to

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<sup>1</sup> The abbreviations used are: PLC, phospholipase C; PI, phosphatidylinositol; PH, pleckstrin homology; Btk, Bruton's tyrosine kinase; GFP, enhanced green fluorescent protein; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; PKC, protein kinase C; GST, glutathione S-transferase.

PI(4,5)P<sub>2</sub>. This methodology also allows visualization of dynamic changes in 3-phosphorylated inositides in single living cells.

#### EXPERIMENTAL PROCEDURES

**Materials**—myo-[<sup>3</sup>H]inositol (80 Ci/mmol) was purchased from Amersham Pharmacia Biotech. EGF (recombinant human) and PDGF-AB (recombinant human) were obtained from Life Technologies, Inc. Ionomycin, LY 294002, bisindolylmaleimide I, and BAPTA were purchased from Calbiochem, and wortmannin was a gift from Kyowa Hakko Laboratories (Tokyo, Japan). All other chemicals were of high pressure liquid chromatography or analytical grade.

**Plasmid Constructs**—The PH domains of PLCδ<sub>1</sub> (amino acids 1–170) and of Bruton's tyrosine kinase (amino acids 1–177) were amplified with the Advantage KlenTaq polymerase mixture (CLONTECH) from human cDNAs (Marathon cDNA from brain and K562 leukemia cells, CLONTECH) with the following primer pairs: PLCδ, 5'-GGCATG-GACTCGGGCCGGGACTTCCTG-3' and 5'-AAGATCTTCCGGGCAT-AGCTGTGCG-3'; Btk, 5'-CCAAGTCCTGGCATCTCAATGCATCTG-3' and 5'-TGGAGACTGGTGTGCTGCTGGCTC-3'. The amplified products were subcloned into the pGEM-Easy T/A cloning vector (Promega) and sequenced with dideoxy sequencing (ThermoSequenase, Amersham Pharmacia Biotech). A second amplification reaction was performed from these plasmids with nested primers that contained restriction sites for appropriate cloning into the pEGFP-N1 plasmid (CLONTECH) to preserve the reading frame. Plasmids were transfected into COS-7 or NIH 3T3 cells and analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blotting for the presence of the GFP fusion constructs using a polyclonal antibody against GFP (CLONTECH).

Mutations were created with the QuickChange<sup>TM</sup> mutagenesis kit (Stratagene). All mutations were confirmed by dideoxy sequencing, and the expression of the protein was confirmed by Western blotting.

**Transfection of Cells for Confocal Microscopy**—Cells were plated onto poly-L-lysine-coated 30-mm diameter circular glass coverslips at a density of  $5 \times 10^4$  cells/dish and cultured for 3 days before transfection with plasmid DNAs (1 μg/ml) using the LipofectAMINE reagent (10 μg/ml; Life Technologies, Inc.) and Opti-MEM (Life Technologies, Inc.). Forty-eight hours after transfection, cells were washed twice with a modified Krebs-Ringer buffer containing 120 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl<sub>2</sub>, 0.7 mM MgSO<sub>4</sub>, 10 mM glucose, and 10 mM Na-Hepes, pH 7.4, and the coverslip was placed into a chamber that was mounted on a heated stage with the medium temperature kept at 33 °C. Cells were incubated in 1 ml of the Krebs-Ringer buffer, and stimuli were added in 0.5 ml of prewarmed buffer after removing 0.5 ml of medium from the cells. Cells were examined in an inverted microscope under a 40× oil immersion objective (Nikon Inc.) and a Bio-Rad laser confocal microscope system (MRC-1024) with Lasersharp acquisition software (Bio-Rad).

**Analysis of Inositol Phosphates in COS-7 Cells**—Inositol phosphates were analyzed in COS-7 cells transfected with the AT<sub>1a</sub> angiotensin II receptor together with selected PH domain-GFP fusion constructs after labeling with myo-[<sup>3</sup>H]inositol for 24 h as described previously (16, 17). <sup>3</sup>H-labeled inositol phosphates were analyzed with Dowex minicolumns.

#### RESULTS

**Distribution of PH Domains Fused to the Enhanced Green Fluorescent Protein**—To follow the localization of isolated PH domains within intact cells, they were fused to GFP and expressed in NIH 3T3 cells. As shown in Fig. 1A (left panel), expressed GFP (without any attached sequence) was found to be cytosolic and was also present in the nucleus. Addition of the Btk PH domain (amino acids 1–177) to the N terminus of GFP had no effect on its localization in quiescent cells (after serum deprivation) and was indistinguishable from GFP alone (Fig. 1A, center panel). In contrast, fusion of the PLCδ PH domain to GFP caused prominent membrane localization of the construct due to its interaction with membrane PI(4,5)P<sub>2</sub> (18) (Fig. 1A, right panel). These results suggest that, unlike the PH domain of PLCδ, the PH domain of Btk does not possess high enough affinity to interact with PI(4,5)P<sub>2</sub> so that it would be sufficient for its membrane targeting.

**Stimulation of Cells Causes Translocation of the Btk PH Domain to Membranes**—Next we examined whether stimulation of cells with growth factors that are known to activate PI

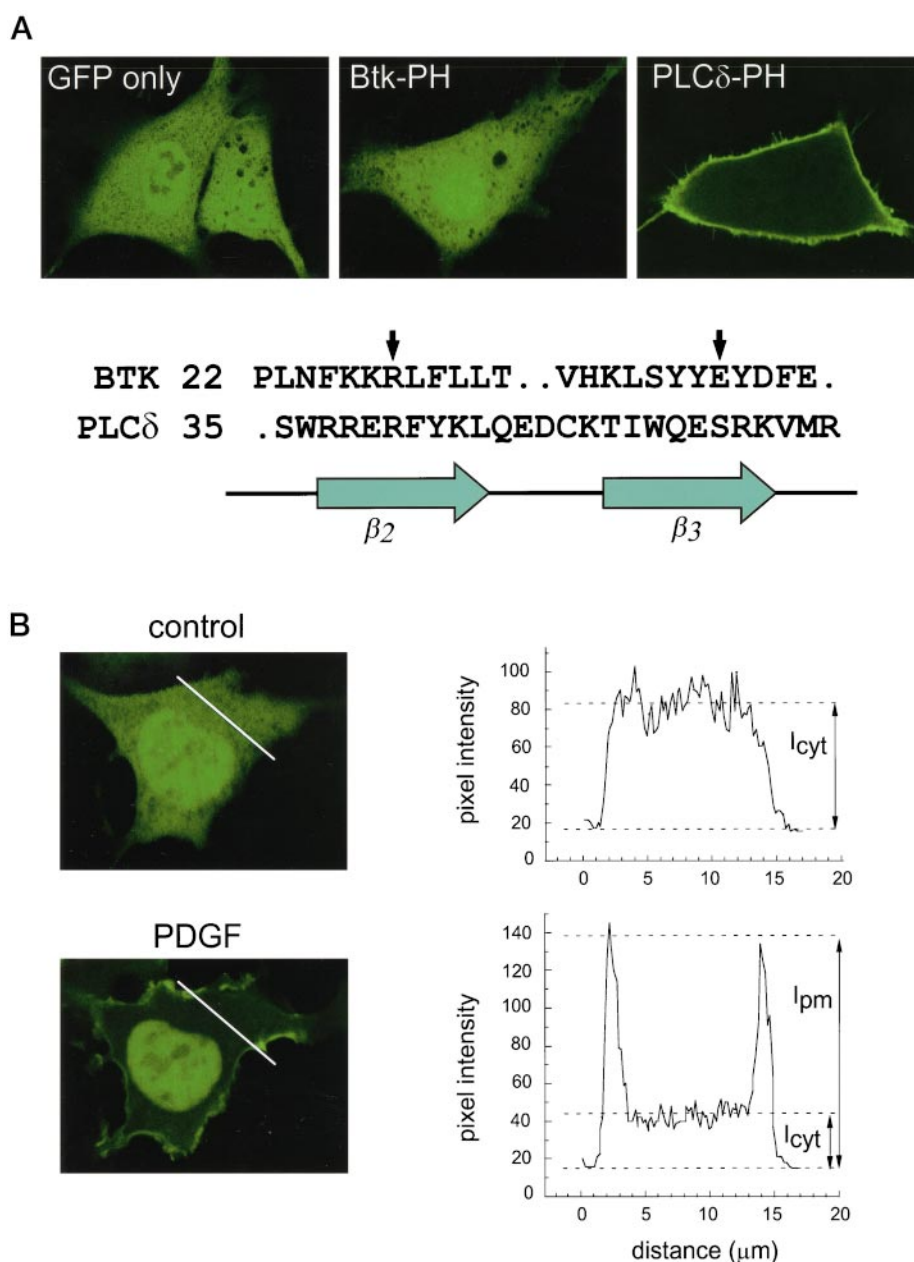
3-kinases and to increase formation of PI(3,4,5)P<sub>3</sub> causes any change in the distribution of the BtkPH-GFP protein. NIH 3T3 cells were transfected with the BtkPH-GFP construct alone or together with the cDNA encoding the human EGF receptor and were stimulated either with PDGF (25–100 ng/ml) (to activate the endogenous PDGF receptor) or with EGF (100 ng/ml) after serum starvation. As shown in Figs. 1B and 2, stimulation of either receptor caused a translocation of the cytosolic fluorescence to the plasma membrane. The decrease in cytosolic fluorescence was most obvious when compared with the bright nuclear signal that did not change in intensity following stimulation in the NIH 3T3 cells. This was in contrast with our previous finding in the same cell type, where the nuclear intensity of the PLCδ PH domain-GFP fusion protein slowly followed the cytoplasmic changes, although with a slight delay (19).

The redistribution of fluorescence was clearly demonstrated by comparing line intensity histograms calculated at selected "cross-sections" of the cell. Dividing the fluorescence intensity of the plasma membrane ( $I_{pm}$ ) by that of the cytosol ( $I_{cyt}$ ) yields a ratio that can be used as an index of membrane localization (Fig. 1B, right panels).

To determine whether a similar translocation of BtkPH-GFP takes place in response to stimulation of other receptors that activate PI 3-kinases, NIH 3T3 cells were also stimulated with EGF (via expressed EGF receptors). EGF stimulation also evoked a translocation response, and we could not detect a notable difference in the localization of the fluorescent signal whether PDGF or EGF was used as the agonist (Fig. 2, A and B). To examine the effects of insulin, another known activator of PI 3-kinases, immortalized hepatocytes (20) were transfected with the BtkPH-GFP construct and subjected to a 6–12-h serum deprivation. Addition of insulin (100 nM) to such cells also stimulated the translocation of the fluorescent signal to the plasma membrane. Interestingly, in this case, localization of the construct to intracellular vesicular structures that moved toward the plasma membrane was also observed. It is also noteworthy that, in the hepatocytes, the nuclear signal intensity decreased in parallel with the insulin-induced membrane localization (Fig. 2C).

**Membrane Localization of BtkPH-GFP Depends on Increased PI 3-Kinase Activity and Membrane PI(4,5)P<sub>2</sub> Levels**—To determine whether the Btk PH domain binds to PI(3,4,5)P<sub>3</sub>, the lipid product of PI 3-kinase, we used the PI 3-kinase inhibitors wortmannin and LY 294002 to prevent formation of the lipid in growth factor-stimulated cells. Addition of wortmannin or LY 294002 to cells after EGF or PDGF stimulation caused dissociation of BtkPH-GFP from the plasma membrane (Fig. 3, A and C). This finding suggested that the Btk PH domain localizes to the membrane by a mechanism that requires the sustained activity of PI 3-kinase(s) and supports the notion that PI(3,4,5)P<sub>3</sub> is the membrane component to which the PH domain of Btk binds in living cells. These results also show that PI(3,4,5)P<sub>3</sub> is actively dephosphorylated during stimulation since, after blocking its increased synthesis, its level rapidly decreases as indicated by the dissociation of BtkPH-GFP from the plasma membrane.

The finding that the maintenance of PI(3,4,5)P<sub>3</sub> levels requires the sustained phosphorylation of PI(4,5)P<sub>2</sub> by PI 3-kinases prompted us to investigate whether decreasing the level of PI(4,5)P<sub>2</sub> has an indirect effect on the localization of the Btk PH domain. Addition of ionomycin (which elevates intracellular [Ca<sup>2+</sup>]) and thereby induces hydrolysis of PI(4,5)P<sub>2</sub> to EGF-stimulated cells caused the rapid release of the fluorescence from the membranes back to the cytosol (data not shown). Since PI(3,4,5)P<sub>3</sub> is not hydrolyzed directly by any known PLC, these



**FIG. 1. Localization of the PH domains of Btk and PLC $\delta$  in transfected NIH 3T3 cells.** *A*, the PH domain of Btk (amino acids 1–177) or PLC $\delta$  (amino acids 1–170) was fused to the N terminus of GFP, and the respective constructs were transiently expressed in NIH 3T3 cells. Confocal images were taken from serum-starved (>5 h) cells incubated at 33 °C. Alignment of the protein sequences within the PH domains (covering the  $\beta_2$ - and  $\beta_3$ -strands) of Btk and PLC $\delta$  is shown. The arrows indicate the positions of the mutations analyzed in this study. *B*, redistribution of fluorescence in NIH 3T3 cells after a 5-min stimulation with 50 ng/ml PDGF (*left panels*). The fluorescent intensity changes across the *white lines* were plotted as line intensity histograms in the *right panels*. Calculation of  $I_{pm}/I_{cyt}$  was used to quantitate the extent of membrane localization.

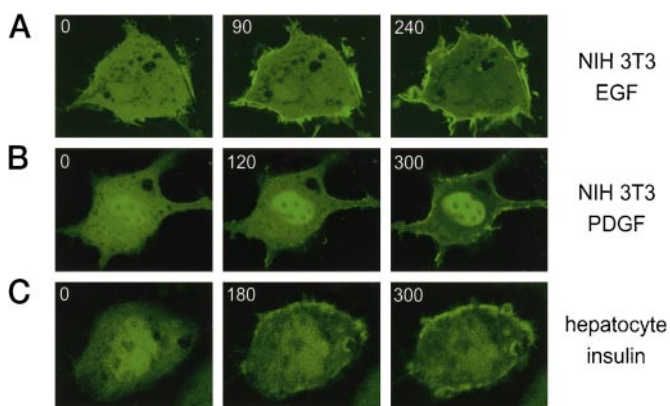
findings are consistent with the sensitivity of PI(3,4,5) $P_3$  levels both to PI 3-kinase activity and to changes in the level of its substrate, PI(4,5) $P_2$ .

**Membrane-targeted PI 3-Kinase Leads to Membrane Localization of the Btk PH Domain**—Next we tested whether cellular production of 3-phosphorylated inositides without receptor stimulation is sufficient to cause the translocation of the Btk PH domain construct to the plasma membrane. For this, we cotransfected NIH 3T3 cells with a membrane-targeted form of PI 3-kinase  $\gamma$  (PI3K $\gamma$ -CAAX) (21) and BtkPH-GFP. Expression of this PI 3-kinase construct has been shown to cause production of PI(3,4,5) $P_3$  and a high level of activation of the Akt protein kinase in COS-7 cells (21). As shown in Fig. 4A, this manipulation yielded cells in which the fluorescent construct was localized to the membranes, but unlike after hormonal stimulation, this localization was not confined to the plasma membrane. In many cells, there were intracellular bright spots that, in some cases, formed “aggregates” with high fluorescent intensities (data not shown). Moreover, very little nuclear localization of fluorescence was observed in these cells. Addition

of wortmannin (300 nM) caused the release of fluorescence from the plasma membrane to the cytosol, but it required a significantly longer time than in cases of acute stimulation by agonists. Also, the spotty intracellular signal appeared to be more resistant to wortmannin treatment.

**Protein Kinase C Activation Is Not a Major Determinant of the Btk PH Domain Translocation Response**—The C1 region of various protein kinase C (PKC) isozymes has been shown to interact with the Btk PH domain based on *in vitro* binding assay (22). This interaction was found to be inhibited by agents that bind either to the C1 domain of PKC (such as phorbol esters) or to the Btk PH domain (such as PI(4,5) $P_2$ ). Moreover, recently, the PI 3-kinase-dependent kinase PDK-1 has been shown to phosphorylate and activate PKC $\zeta$  (23). Therefore, we examined the effect of phorbol esters and PKC inhibitors on the membrane translocation response of the Btk PH domain. Stimulation of NIH 3T3 cells with phorbol esters (12-*O*-tetradecanoylphorbol-13-acetate, 100 nM to 1  $\mu$ M) had no effect on the distribution of BtkPH-GFP (Fig. 4B). Moreover, stimulation with EGF (or PDGF) induced translocation of BtkPH-GFP in



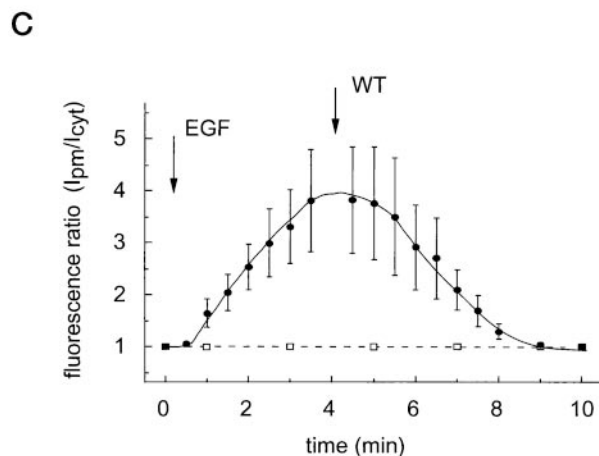
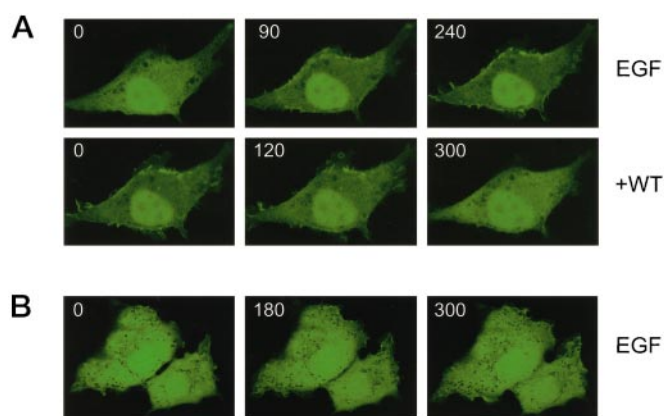


**FIG. 2. Agonist-induced changes in the localization of BtkPH-GFP in NIH 3T3 cells stimulated with EGF or PDGF or in hepatocytes stimulated with insulin.** NIH 3T3 cells were transiently cotransfected with plasmids encoding the human EGF receptor and BtkPH-GFP. Serum-starved cells were stimulated either with EGF (100 ng/ml) (A) or with PDGF (50 ng/ml) (B) at 33 °C. Immortalized hepatocytes were transfected with the BtkPH-GFP construct for 48 h and were stimulated with insulin (100 nM) after serum deprivation at 33 °C (C). Confocal images were taken at every 30 s. The numbers show the time in seconds at which images were recorded. Stimuli were added at 15 s.

cells that either were pretreated with the PKC inhibitor bisindolylmaleimide I (Gö 6850, up to 500 nM, 10 min) (Fig. 4B) or were treated with 100 nM phorbol 12-myristate 13-acetate for 12 h before PDGF stimulation (data not shown). Similarly, addition of the PKC inhibitor to PDGF-stimulated cells (after Btk PH domain translocation to the membrane had taken place) had no significant effect on the distribution of fluorescence, whereas subsequent addition of wortmannin rapidly released the fluorescence from the membranes (data not shown). (The PKC inhibitor applied at this concentration reversed the effects of phorbol 12-myristate 13-acetate on a variety of cell responses in our laboratory.) Although these data cannot rule out PKC being a regulator of the holoprotein Btk, they did not suggest an important role of PKC in the PI 3-kinase-mediated membrane targeting of the Btk PH domain.

**Mutations within the Btk PH Domain Affect Its Ability to Localize to the Membrane**—Several mutations of Btk that are responsible for X-linked agammaglobulinemia are located within the PH domain of the protein and impair its membrane association (11). Therefore, we examined the ability of the best characterized mutant, R28C (which is unable to bind  $\text{PI}(3,4,5)\text{P}_3$  *in vitro* (6, 8)), to localize to the membrane in living cells in response to stimulation. As shown in Fig. 3 (B and C), no membrane localization of the R28C mutant of BtkPH-GFP was observed after PDGF or EGF stimulation in NIH 3T3 cells (or in COS-7 cells; data not shown).

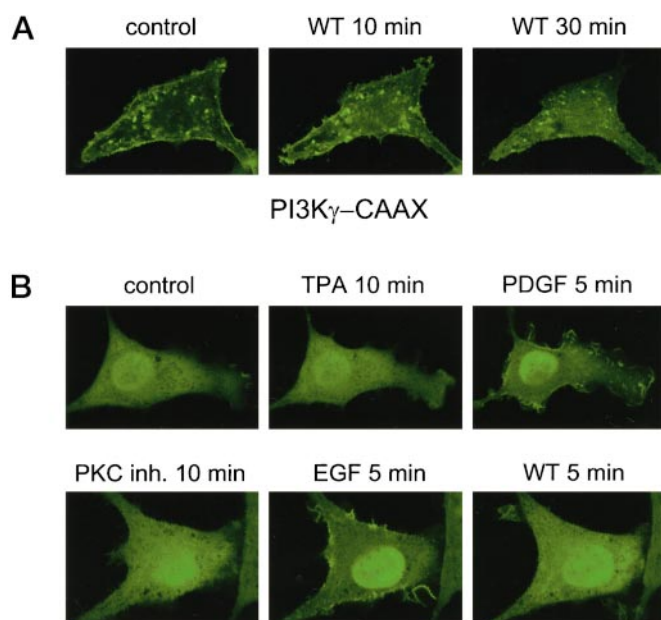
The E41K mutation, which causes transformation in NIH 3T3 cells and shows enhanced membrane association (15), was also introduced into the BtkPH-GFP construct, and its localization within intact cells was examined. As shown in Fig. 5A, the E41K mutant displayed significant membrane localization even in quiescent NIH 3T3 cells and still displayed translocation in response to EGF (100 ng/ml). Application of wortmannin (300 nM) reversed the EGF-induced increase in membrane association, but did not reduce it below its initial level. Wortmannin also failed to affect the basal localization of the construct when added to cells that were not stimulated with EGF (data not shown). In view of the ability of the E41K mutant to associate with membranes of unstimulated cells, which contain only small amounts (if any) of  $\text{PI}(3,4,5)\text{P}_3$ , we investigated the possibility that this mutant BtkPH-GFP has a diminished binding specificity for  $\text{PI}(3,4,5)\text{P}_3$  and hence is also able to bind



**FIG. 3. EGF-induced changes in the localization of the wild-type and R28C mutant BtkPH-GFP constructs in transfected NIH 3T3 cells.** NIH 3T3 cells expressing the human EGF receptor together with wild-type BtkPH-GFP (A) or the R28C mutant of BtkPH-GFP (B) were stimulated with 100 ng/ml EGF after serum starvation. Wortmannin (WT; 300 nM) was added 4 min after EGF (A). Experiments were carried out at 33 °C, and confocal images were taken at every 30 s. The numbers indicate the elapsed time in seconds. Fluorescence ratios calculated from line intensity plots (see legend to Fig. 1) obtained from each of the frames were plotted against time (C). ●, wild-type BtkPH-GFP (means  $\pm$  S.E.,  $n$  = five cells quantitated); □, R28C mutant (based on a large number of cells observed in several independent experiments).

to  $\text{PI}(4,5)\text{P}_2$  that is present in membranes of unstimulated cells. As shown in Fig. 5A (panels d–h),  $\text{PI}(4,5)\text{P}_2$  breakdown evoked by the addition of the  $\text{Ca}^{2+}$  ionophore ionomycin caused a complete release of the membrane-bound fluorescence into the cytosol, whereas subsequent chelation of  $\text{Ca}^{2+}$  by BAPTA caused a reappearance of the signal at the plasma membrane. The effect of ionomycin was inhibited by preincubation with 10 mM neomycin (to inhibit PLC; data not shown). The relocation of fluorescence after  $\text{Ca}^{2+}$  chelation was not prevented by 300 nM wortmannin, but was abolished by the addition of 100  $\mu\text{M}$  quercetin, which inhibits  $\text{PI}(4,5)\text{P}_2$  resynthesis (19) (Fig. 5B). These changes were very similar to those observed with the PLC $\delta$  PH domain-GFP construct (which binds to  $\text{PI}(4,5)\text{P}_2$  (18, 19, 24)) and are consistent with the assumption that the E41K mutation impairs the ability of the Btk PH domain to discriminate between  $\text{PI}(3,4,5)\text{P}_3$  and  $\text{PI}(4,5)\text{P}_2$  effectively.

**The E41K Mutant, but Not the Wild-type or R28C Mutant Btk PH Domain, Interferes with Agonist-induced Hydrolysis of  $\text{PI}(4,5)\text{P}_2$** —To further investigate whether the E41K mutant Btk PH domain is able to bind to membrane  $\text{PI}(4,5)\text{P}_2$  within the intact cell, we examined the ability of this construct to

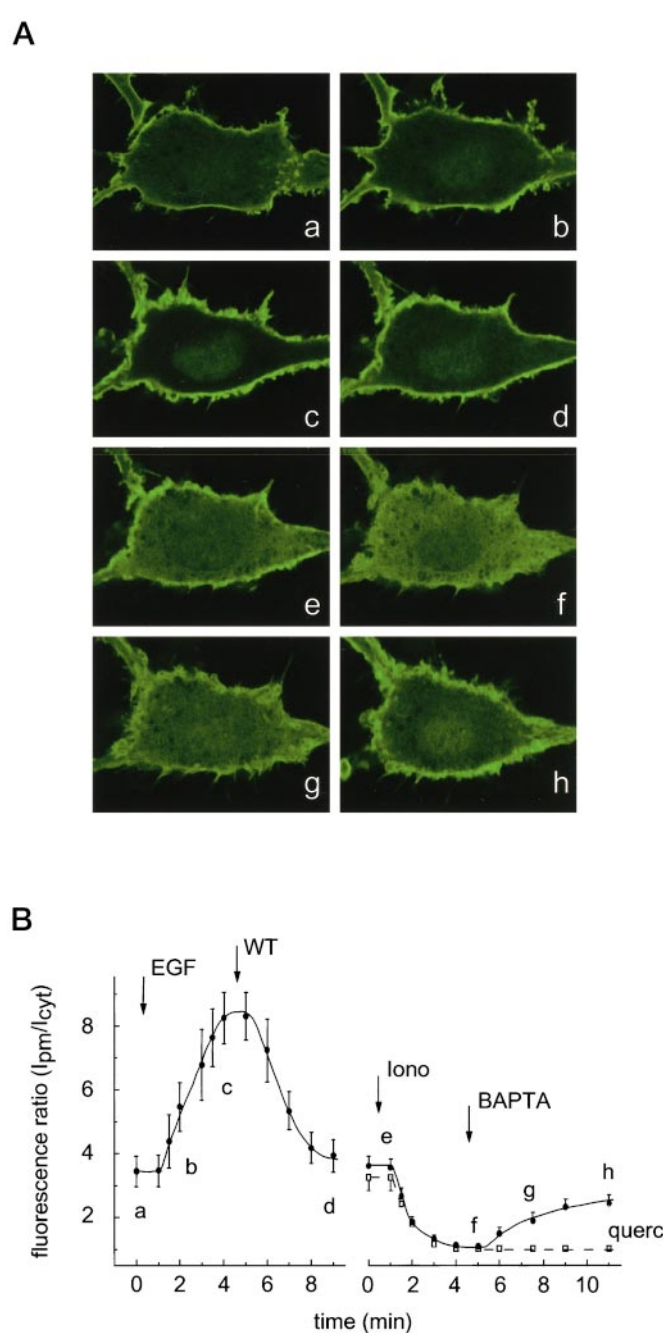


**FIG. 4. Effects of membrane-targeted PI 3-kinase  $\gamma$  (PI3K $\gamma$ -CAAX) and PKC activation on the localization of BtkPH-GFP in NIH 3T3 cells.** A, NIH 3T3 cells were cotransfected with BtkPH-GFP and a membrane-targeted version of PI 3-kinase  $\gamma$  (21). Addition of 300 nM wortmannin (WT) slowly reversed plasma membrane localization. B, NIH 3T3 cells coexpressing BtkPH-GFP and the human EGF receptor were stimulated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 200 nM) and subsequently with 50 ng/ml PDGF (upper panels) or with 100 ng/ml EGF after pretreatment with 500 nM bisindolylmaleimide I (lower panels). Shown are representative cells from several independent observations. *inh.*, inhibitor.

interfere with agonist-induced PI(4,5) $P_2$  hydrolysis. As shown earlier for the PLC $\delta$  PH domain (25) and for the pleckstrin PH domain (26) (both of which bind to PI(4,5) $P_2$ ), expression of these molecules inhibited receptor-mediated inositol phosphate production, presumably by interfering with PLC-mediated hydrolysis of PI(4,5) $P_2$ . As shown in Fig. 6, neither the wild-type Btk PH domain nor the R28C mutant had a significant effect on angiotensin II-stimulated inositol phosphate production in COS-7 cells expressing the AT $_{1a}$  receptor together with the GFP construct of interest. In contrast, E41K mutant BtkPH-GFP showed a significant inhibitory effect, although this inhibition was not as strong as that caused by expression of PLC $\delta$  PH domain-GFP (Fig. 6).

#### DISCUSSION

The important role of the Btk PH domain was originally recognized after identifying and analyzing mutations that cause X-linked agammaglobulinemia in humans (14). Several (although not all) of these mutations are within the PH domain of Btk. Some mutations cause a folding defect, and others affect its function, presumably interfering with the binding characteristics of this module (27). The ability of Btk to interact with membranes appears to be the key regulatory element in determining the function(s) of the kinase, and several lines of evidence suggest that the PH domain is a critical region of the molecule for membrane association (27). Although  $\beta\gamma$ -subunits of heterotrimeric G-proteins (28) and various PKC isoforms (29) have been shown to interact with the PH domain of Btk, more recent studies indicate that the 3-phosphorylated inositides, PI(3,4,5) $P_3$  in particular, are its binding partners in the membrane. The isolated recombinant PH domain of Btk in the form of a GST fusion protein has been demonstrated to bind PI(3,4,5) $P_3$  in a BIAcore assay system (6) or by utilizing lipid micelles *in vitro* (8). Binding of the lipid to the PH domain of Btk was found to depend on the ionic composition (6), and the



**FIG. 5. Effects of EGF and ionomycin on the localization of the E41K mutant of the BtkPH-GFP construct in transfected NIH 3T3 cells.** NIH 3T3 cells expressing the human EGF receptor and the E41K mutant of BtkPH-GFP were stimulated with EGF (100 ng/ml) and/or ionomycin (10  $\mu$ M). Shown in A are confocal images taken at selected times during the experiment from a representative cell taken through a full protocol. Shown in B (left panel) are fluorescence ratios (calculated and plotted as described in the legend to Fig. 1) from cells stimulated with EGF followed by the addition of wortmannin (WT; 300 nM) ( $n = 3$ ). Cells stimulated with ionomycin (Iono) after a 10-min treatment with 300 nM wortmannin followed by the addition of BAPTA (2 mM) ( $n = 6$ ) are shown in B (right panel). Dashed lines represent cells that were pretreated with quercetin (querc; 100  $\mu$ M, 10 min) before addition of ionomycin to inhibit PI(4,5) $P_2$  resynthesis ( $n = 3$ ). The letters on the images correspond to the letters on the fluorescence ratio-time plot, to indicate the time of the recording.

fatty acid side chains of PI(3,4,5) $P_3$  were also shown to be important for the interaction (8). In contrast, soluble inositol 1,3,4,5-tetrakisphosphate was found to bind to the Btk PH domain with high affinity in one report (30). These *in vitro* studies also showed that the binding of the Btk PH domain to



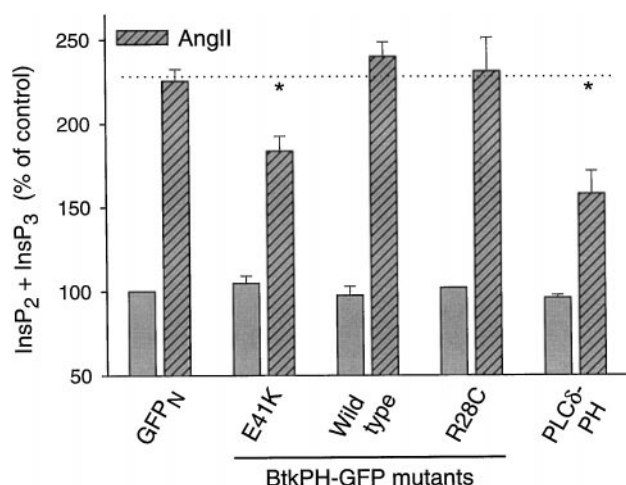


FIG. 6. Effects of expressed PH domains on angiotensin II-induced formation of inositol phosphates in COS-7 cells. COS-7 cells coexpressing the AT<sub>1a</sub> angiotensin II (*Ang II*) receptor and the respective PH domain-GFP constructs were labeled with *myo*-[<sup>3</sup>H]inositol (20  $\mu$ Ci/ml, 24 h) and stimulated with 1  $\mu$ M angiotensin II in the presence of 10 mM LiCl for 30 min. Inositol phosphates were extracted and separated by Dowex AG 1-X8 minicolumns. Means  $\pm$  S.E. from four separate experiments, each performed in duplicate, are shown. \*, significant difference from the control response ( $p < 0.001$ ). *Ins*, inositol; GFP<sub>N</sub>, pEGFP-N1 plasmid.

PI(4,5)P<sub>2</sub> is much weaker, providing the specificity that would be required for its general regulation by 3-phosphorylated inositides. In all of these studies, the R28C mutant PH domain, which causes X-linked immunodeficiency in mice, was found to be unable to bind PI(3,4,5)P<sub>3</sub>.

In this report, we provide evidence that the isolated PH domain of Btk fused to the fluorescent reporter molecule GFP exhibits agonist-dependent membrane association when expressed in a variety of cells. This method allowed analysis of the binding specificity as well as imaging of spatiotemporal changes in the membrane association of this protein module inside single living cells. Our results show that the Btk PH domain does not localize to membranes inside quiescent cells (suggesting that its binding to PI(4,5)P<sub>2</sub> is probably too weak for such membrane targeting) and that the agonist-induced translocation of BtkPH-GFP to membranes is dependent on PI 3-kinase activation as well as on membrane PI(4,5)P<sub>2</sub> levels. Moreover, the production of PI(3,4,5)P<sub>3</sub> by membrane-targeted PI 3-kinase was sufficient to target the Btk PH domain to membranes. These results, together with *in vitro* binding data (6, 8), further support the idea that the Btk PH domain binds to PI(3,4,5)P<sub>3</sub> of the plasma membrane and that this mechanism is sufficient to regulate membrane localization of the protein.

These results do not rule out the possibility that additional factors (proteins) participate in the membrane anchoring of the Btk PH domain, similarly to the protein anchors (RACK proteins) (31) that stabilize the various motifs of PKC. Similarly, even though the Akt kinase has a PH domain that appears to be sufficient to localize this protein to 3-phosphorylated inositides (32), its activation requires PDK-1, a kinase that also possesses a PH domain and that also is regulated by 3-phosphorylated lipids (33). Although *in vitro* studies suggested that the C1 domain of various PKC isozymes can interact with the Btk PH domain (22), our studies in intact cell did not provide evidence for a role of PKC in the membrane targeting of the Btk PH domain. Based on the studies of Yao *et al.* (22), the association of the Btk PH domain with PKCs would be the strongest in quiescent cells, and either the binding of diacylglycerol (or phorbol 12-myristate 13-acetate) to PKC or the inositol lipid head group to the Btk PH domain (*i.e.* activation of the respec-

tive enzymes) would eliminate this association. Certainly, stimulation with phorbol 12-myristate 13-acetate, which has been widely demonstrated to induce membrane translocation of various PKC isozymes (34, 35), failed to induce membrane translocation of the Btk PH domain in the present study, and a PKC inhibitor did not affect translocation induced by PDGF. The existence of additional factors contributing to the membrane localization of PH domains has been suggested by recent studies in which the cytohesin-1 PH domain was shown to require a small basic flanking sequence to effectively localize it to membranes and to exert a dominant-negative effect on cell adhesion (36). In this context, it is important to note that our Btk PH domain construct also contained the small adjacent Btk motif that had been found important for proper folding in bacteria (27). Clearly, more studies are needed to fully explore the complexity of inositol lipid-PH domain interactions that regulate Btk and other PI 3-kinase-regulated effectors.

Introduction of the R28C mutation into the BtkPH-GFP construct prevented its membrane localization in response to stimulation, in agreement with the data regarding the diminished affinity of this mutant for PI(3,4,5)P<sub>3</sub> (6, 8, 30). Arg-28 is located within the predicted inositol-binding pocket of the Btk PH domain and, based on structural alignments, corresponds to Arg-40 of the PLC $\delta$  PH domain. Mutation of this residue in the latter molecule (which makes contact with the 5-phosphate of PI(4,5)P<sub>2</sub> (24)) prevents its binding to PI(4,5)P<sub>2</sub> and its membrane association (18, 19). Based on this analogy, it is expected that replacing the positively charged Arg-28 with a non-charged residue would result in the loss of binding affinity for PI(3,4,5)P<sub>3</sub>.

Another mutation within the Btk PH domain, E41K (15), was found to show increased membrane localization in quiescent cells and further translocation in response to EGF stimulation. Although there are a number of reasons why this mutant protein could bind to the membrane (including a higher affinity for PI(3,4,5)P<sub>3</sub>), its similar behavior compared with the PLC $\delta$  PH domain raised the possibility that it also binds to membrane PI(4,5)P<sub>2</sub>. Membrane association of the E41K mutant of BtkPH-GFP was not abolished by PI 3-kinase inhibitors, but showed correlation with PI(4,5)P<sub>2</sub> levels after manipulations of the latter by Ca<sup>2+</sup> ionophores, Ca<sup>2+</sup> chelators, and inhibitors of PI(4,5)P<sub>2</sub> resynthesis. Also, it showed a significant inhibitory effect on agonist-induced PI(4,5)P<sub>2</sub> hydrolysis, a feature of PH domains that bind PI(4,5)P<sub>2</sub>. Comparison of the crystal structure of PLC $\delta$  and Btk reveals that Glu-41 is located in a position that corresponds to a region of the PLC $\delta$  PH domain (Ser-55 and Arg-56) that makes important contacts with the phosphates at the 4- and 5-positions of the inositol ring in PI(4,5)P<sub>2</sub> (24). An acidic amino acid (Glu-41) in this position could provide a significant repulsive force to prevent association of the mutant protein with PI(4,5)P<sub>2</sub>. Mutation of Glu-41 to Lys would therefore be expected to increase PI(4,5)P<sub>2</sub> binding. Indeed, an analogous mutation within the PH domain of PLC $\delta$  (E54K) has been reported to enhance the catalytic activity of the enzyme, presumably by increasing its affinity to PI(4,5)P<sub>2</sub> (37). Such an affinity increase in the E41K mutant of Btk toward both PI(4,5)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> could explain why the E41K substitution did not significantly affect the ability of Ins(1,4,5)P<sub>3</sub> to displace Ins(1,3,4,5)P<sub>4</sub> in the binding studies performed on the isolated BtkPH-GST fusion protein (30).

Members of the Tec tyrosine kinase family have been recognized recently as important modulators of Ca<sup>2+</sup> influx pathways in B- and T-lymphocytes (38) via a mechanism that amplifies inositol 1,4,5-trisphosphate formation after PLC $\gamma$  activation (39, 40). This function of the kinases also relies upon the interaction of their PH domains with membrane PI(3,4,5)P<sub>3</sub>

(39). However, it is also important to note that the present results only explore the aspect of Btk function from the standpoint of its PH domain and that additional interactions mediated by other domains of the molecule may greatly affect the overall localization of the holoprotein. Nevertheless, the ability of the isolated Btk PH domain to confer PI(3,4,5)P<sub>3</sub>-dependent membrane localization may also be utilized as a probe that can detect changes in the level of this lipid in single living cells with fine spatial resolution. Such a feature of other PH domain-GFP fusion constructs that can interact with PI(3,4,5)P<sub>3</sub> has been recently demonstrated in insulin-stimulated adipocytes (41) and EGF-stimulated PC-12 cells (42).

In summary, we have shown that the isolated PH domain of Btk interacts with plasma membranes with characteristics that are consistent with its binding to membrane PI(3,4,5)P<sub>3</sub>. This interaction appears to be a fundamental aspect of the Btk protein that is severely compromised in a human disease and can now be monitored in single living cells. This methodology will also help to better understand the role of inositide phospholipids in membrane-protein interactions.

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